Flunarizine Prevents Hepatitis C Virus Membrane Fusion in a Genotype-dependent Manner by Targeting the Potential Fusion Peptide within E1

<table>
<thead>
<tr>
<th>Journal:</th>
<th>Hepatology</th>
</tr>
</thead>
<tbody>
<tr>
<td>Manuscript ID:</td>
<td>HEP-15-0888.R1</td>
</tr>
<tr>
<td>Wiley - Manuscript type:</td>
<td>Original</td>
</tr>
<tr>
<td>Date Submitted by the Author:</td>
<td>07-Jul-2015</td>
</tr>
</tbody>
</table>
| Complete List of Authors: | Perin, Paula; Twincore, Experimental Virology  
Haid, Sibylle; Twincore, Experimental Virology  
Brown, Richard; Twincore, Experimental Virology  
Doerrbecker, Juliane; Twincore, Experimental Virology  
Schulze, Kai; Helmholtz Centre of Infection Research, Department of Vaccinology and Applied Microbiology  
Zeilinger, Carsten; Leibniz Universität, Institute of Organic Chemistry and Center of Biomolecular Drug Research  
von Schaewen, Markus; Princeton University, Molecular Biology  
Heller, Brigitte; Princeton University, Department of Molecular Biology  
Vercauteren, Koen; Ghent University, clinical biology  
Luxenburger, Eva; Helmholtz Institute for Pharmaceutical Research, Baktash, Yasmine; The University of Chicago, Department of Microbiology  
Vondran, Florian; Medizinische Hochschule Hannover, Visceral- and Transplantation Surgery  
Speerstra, Sietkse; University of Alberta, Department of Biochemistry; University of Alberta, Department of Medical Microbiology and Immunology  
Awadh, Abdullah; University of Alberta, Department of Biochemistry; University of Alberta, Department of Medical Microbiology and Immunology  
Mukhtarov, Furkat; University of Alberta, Department of Biochemistry; University of Alberta, Department of Medical Microbiology and Immunology  
Schang, Luis; University of Alberta, Department of Biochemistry; University of Alberta, Department of Medical Microbiology and Immunology  
Kirschning, Andreas; Leibniz Universität, Institute of Organic Chemistry and Center of Biomolecular Drug Research  
Müller, Rolf; Helmholtz Institute for Pharmaceutical Research, Guzman, Carlos; Helmholtz Centre for Infection Research, Vaccinology; Kaderali, Lars; Technische Universität Dresden, Institute for Medical Informatics and Biometry  
Randall, Glenn; University of Chicago, Microbiology; Meuleman, Philip; Ghent University and Hospital, Center for Vaccinology; Ploss, Alexander; Princeton University, Department of Molecular Biology; Pietschmann, Thomas; Twincore, Experimental Virology |
| Keywords: | diphenylmethylpiperazine, phenothiazines, diarylbutylpiperidine, E1 fusion peptide, cholesterol |
Flunarizine Prevents Hepatitis C Virus Membrane Fusion in a Genotype-dependent Manner by Targeting the Potential Fusion Peptide within E1

Paula M. Perin¹, Sibylle Haid¹, Richard J. P. Brown¹, Juliane Doerrbecker¹, Kai Schulze², Carsten Zeilinger³, Markus von Schaewen⁴, Brigitte Heller⁴, Koen Vercauteren⁵, Eva Luxenburger⁶, Yasmine M. Baktash⁷, Florian W. R. Vondran⁸, Sietkse Speerstra⁹, Abdullah Awadh¹⁰,¹¹, Furkat Mukhtarov⁹,¹¹, Luis M Schang⁹,¹⁰,¹¹, Andreas Kirschning³, Rolf Müller⁶, Carlos A. Guzman², Lars Kaderali¹², Kaderali¹², Glenn Randall⁷, Philip Meuleman⁵, Alexander Ploss⁴, Thomas Pietschmann¹,¹⁰,¹³*

¹Institute of Experimental Virology, TWINCORE, Centre for Experimental and Clinical Infection Research; a joint venture between the Medical School Hannover (MHH) and the Helmholtz Centre for Infection Research (HZI), Hannover, Germany.
²Department of Vaccinology and Applied Microbiology, Helmholtz Centre of Infection Research, Braunschweig, Germany.
³Institute of Organic Chemistry and Center of Biomolecular Drug Research (BMWZ), Leibniz Universität, Hannover, Germany.
⁴Department of Molecular Biology, Princeton University, Princeton, United States.
⁵Center for Vaccinology, Ghent University, Ghent, Belgium.
⁶Helmholtz Institute for Pharmaceutical Research Saarland, Saarbrücken, Germany.
⁷Department of Microbiology, The University of Chicago, Chicago, United States.
⁸ReMediES, Department of General, Visceral and Transplantation Surgery, Hannover Medical School, Hannover, Germany.
⁹Technical University Dresden, Dresden, Germany.
Key words:
diphenylmethylpiperazine, phenothiazines, diarylbutylpiperidine, E1 fusion peptide, cholesterol.

Conflict of interest:
The authors do not have a conflict of interest.

*Address for correspondence:
Prof. Dr. rer. nat. Thomas Pietschmann
Institute of Experimental Virology
Twincore, Centre for Experimental and Clinical Infection Research
Feodor-Lynen Str 7-9
30625 Hannover
Email: thomas.pietschmann@twincore.de
Phone: +49-511-220027130, FAX +49-511-220027139
paula.perin@twincore.de, sibylle.haid@twincore.de, richard.brown@twincore.de,
 juliane.doerrbecker@twincore.de, kai.schulze@helmholtz-hzi.de, zeilinger@biophysik.uni-
hannover.de, schaewen@princeton.edu, bh6@princeton.edu, koen.vercauteren@ugent.be,
eva.luxenburger@helmholtz-hzi.de, yasmine.margaret@gmail.com, vondran.florian@mh-
hannover.de, speerstr@ualberta.ca, awadh@ualberta.ca, furkat@ualberta.ca, lschang@ualberta.ca, andreas.kirschning@oci.uni-hannover.de, rolf.mueller@helmholtz-hzi.de, carlos.guzman@helmholtz-hzi.de, lars.kaderali@tu-dresden.de, grandall@bsd.uchicago.edu, philip.meuleman@ugent.be, aploss@Princeton.EDU

List of Abbreviations:

HCV: Hepatitis C Virus
GTs: Genotypes
G-Luc: Gaussia luciferase
F-Luc: Firefly luciferase
HCVpp: HCV pseudoparticles
HCVcc: cell culture-derived HCV
PHH: Primary human hepatocyte

Financial Support

This work was funded by a grant from the Helmholtz-Alberta Initiative (HAI-IDR) to T.P. P.M.P. was funded by a scholarship of the Hannover biomedical research school within the centre for infection biology (ZIB-program). The work was also supported by grants from the National Institute for Allergy and Infectious Diseases (5R01AI107301 to AP) and from the Canadian Institute for Health Research (CIHR MOP130333 to LMS). M.v.S. is a recipient of a postdoctoral fellowship from the German Research Foundation.
Abstract

To explore mechanisms of hepatitis C virus (HCV) replication we screened a compound library including licensed drugs. Flunarizine, a diphenylmethylpiperazine used to treat migraine, inhibited HCV cell entry in vitro and in vivo in a genotype-dependent fashion. Analysis of mosaic viruses between susceptible and resistant strains revealed that E1 and E2 glycoproteins confer susceptibility to flunarizine. Time of addition experiments and single particle tracking of HCV demonstrated that flunarizine specifically prevents membrane fusion. Related phenothiazines and pimozide also inhibited HCV infection and preferentially targeted HCV genotype 2 viruses. However, phenothiazines and pimozide exhibited improved genotype coverage including the difficult to treat genotype 3. Flunarizine-resistant HCV carried mutations within the alleged fusion peptide and displayed cross-resistance to these compounds, indicating that these drugs have a common mode of action. Conclusion: These observations reveal novel details about HCV membrane fusion. Moreover, flunarizine and related compounds represent first-in-class HCV fusion inhibitors that merit consideration for repurposing as cost-effective component of HCV combination therapies.

Introductory statement

Whole life cycle HCV screen of licensed drugs reveals a class of compounds that inhibit HCV fusion.
Introduction

HCV is a liver-tropic, enveloped virus of the family *Flaviviridae*. It possesses a plus-stranded RNA genome of 9.6 kb which encodes a single polyprotein. Proteolytic processing mediated by cellular and two viral proteases (NS2-3 and NS3-4A) liberates ten distinct HCV polypeptides (1). The structural proteins core, E1 and E2 compose the virion with core encasing the viral RNA and the glycoproteins E1-E2 mediating receptor interactions and low pH triggered membrane fusion. The p7 ion channel is essential for virus assembly and protection of E1-E2 during virus release. The NS3, 4A, 4B, 5A and 5B assemble into a multi-protein complex critical for viral RNA replication. HCV is highly variable and viral strains are classified into seven distinct genotypes (GTs), 67 confirmed and 21 unassigned subtypes (2).

Chronic HCV infection frequently leads to liver fibrosis, cirrhosis and hepatocellular carcinoma and it has become the leading indication for liver transplantation (3). Novel drugs have improved treatment options, and several efficacious combination therapies are available (4). However, these regimens are expensive limiting access particularly in medium to low income countries where HCV is most prevalent (5). To identify alternative, cost-effective treatment options for HCV and to explore mechanisms of viral replication, we screened a compound library including drugs approved to treat neuronal or heart diseases for antiviral activity against HCV.
Material and Methods

Whole life cycle screen: The initial screen of the compounds was performed according to what was previously published (6). Briefly, Huh7-Lunet/hCD81 cells constitutively expressing gaussia luciferase (G-Luc) were transfected with firefly reporter Jc1 (pFK-Luc-Jc1) and seeded into 96-well plates. Media containing serial dilutions of each compound were added to the cells after 4h. After 48h, the supernatant containing G-Luc was collected for cell viability assessment and cells were lysed and measured for firefly luciferase (F-Luc) activity as an indicator of HCV RNA replication. The supernatant of these cells was used to infect target cells. These were lysed after 48h and their reporter levels measured for whole life cycle assessment.

Fusion at the plasma membrane assay: The assay was performed as previously described (7). Huh7-Lunet/hCD81 cells (3 x 10^5 cells/ml) were seeded into each well of a 6-well plate one day before the experiment. The following day, cells were treated with Concanamycin A (5nM) for 1h at 37°C, before infection with concentrated reporter viruses in presence of Concanamycin A. The cells were washed twice with PBS and incubated with medium containing Concanamycin A for 1h at 37°C. Subsequently, cells are incubated for 5 min at 37°C with pH7 or pH5 citric acid buffer (McIlvaine buffer system). Fresh media was added to the cells in continuous presence of Concanamycin A for 3h longer. Medium was changed and infectivity was measured by assessment of reporter activity following 48h.

Virus passaging in presence of flunarizine: Huh7-Lunet/hCD81 cells were seeded in 6-well plates (4 x 10^5 cells/well) one day before being infected with Jc1 virus stocks (pFK-Jc1) for 4h in presence of two concentrations of flunarizine (5.25µM or 10.5µM) or 1% DMSO before addition of fresh medium. 48 to 72h later, cells were split and flunarizine or DMSO was added after 4h. After 48h, virus containing supernatants were used for infection of new Huh7-Lunet-hCD81 cells, according to the procedure performed for the first infection. After 10 cycles of infection of
naïve cells and splitting of virus producing cells (approximately 10 weeks), cells were allowed to
produce viruses in absence of any compound for 16h and supernatant was harvested for core
measurement by ELISA. The stocks from DMSO, flunarizine 5.25µM and flunarizine 10.5µM -
passaged viruses were normalized to have same core levels and used for infection of cells seeded
in cover-slips in 24-well plates. Infection efficiency was assessed by immunofluorescence using
the NS5A-specific monoclonal antibody 9E10 at a dilution of 1:2,000. Bound primary antibodies
were detected using goat anti-mouse IgG-specific secondary antibodies conjugated to Alexa
Fluor 488 (Sigma) at a dilution of 1:1000. Nuclear DNA was stained using DAPI at a dilution of
1:3000.

Results

Flunarizine inhibits HCV entry both in vitro and in vivo in a genotype-dependent fashion. Licensed HCV drugs target either polyprotein processing (NS3-4A protease inhibitors) or RNA replication (NS5A inhibitors, NS5B polymerase inhibitors). To identify molecules that would complement these therapies and that may reveal novel insights into other life cycle steps, we aimed at identifying inhibitors with a novel mode of action. In addition, such molecules should not suffer from potential viral cross-resistance to established drug classes. Thus, we chose a whole life cycle screening assay based on the GT2a chimeric Jc1-luciferase reporter virus (JcR-2a) (8), which interrogates HCV entry, RNA translation, polyprotein processing, RNA replication and virus assembly (Supporting Fig. S1 Fig. and (6)). Since inhibitors of the influenza A virus M2 ion channel have been used to treat humans and because HCV p7 is an ion channel protein essential for virus production, we screened a library of 23 compounds including several clinically approved ion channel inhibitors used to treat heart or neurologic conditions (Supporting Table ST1). Fifteen compounds inhibited HCV entry, assembly or release (Supporting Fig. S1).
Flunarizine displayed an IC$_{50}$ value of 388nM (Supporting Table ST2) and a CC$_{50}$ value of 10.85µM which is equivalent to a therapeutic index (CC$_{50}$/IC$_{50}$) of greater than 25 (Fig. 1A). Flunarizine had recently emerged as an anti-HCV inhibitor in independent screening campaigns (9-11). However, its mode of action remained unclear. Moreover, it was not explored if this molecule is antiviral in primary human hepatocytes and in vivo.

To address these limitations, we inoculated Huh7-Lunet/hCD81/G-Luc cells with a Jc1-F-Luc virus in the presence of the compound and subsequently washed away unbound virus and compound. **Virus-encoded F-Luc activity was decreased by more than 10-fold at a dose of 1.3µM** whereas cell-encoded G-Luc expression was not affected, thereby indicating absence of cytotoxicity (Fig. 1B). In contrast, addition of flunarizine to HCV RNA-transfected cells did not reduce release of infectious virus particle as determined by a core-specific ELISA and limiting dilution assay (TCID$_{50}$) (Supporting Fig. S2). Interestingly, it did not inhibit entry of HCV pseudotypes (HCVpp) carrying the J6-derived glycoproteins (Supporting Figure S3). Thus, flunarizine selectively inhibits entry of authentic, cell culture derived HCV (HCVcc) but not of HCVpp. Moreover, it does not inhibit RNA replication or virus assembly (Fig. 1A and Supporting Fig. S2).

Next, we infected primary human hepatocytes (PHH) with Jc1 particles in the presence of telaprevir, flunarizine or dimethylsulfoxide (DMSO) solvent. Flunarizine significantly inhibited *de novo* production of infectious virus at both 24 and 48h after inoculation compared to inoculation in the presence of solvent (Fig. 1C). Finally, we administered flunarizine to HCV cell entry reporter mice, which express crucial human co-factors for HCV entry and carry a luciferase reporter gene that is activated by the CRE-recombinase (12). When these animals were challenged with infectious Jc1 expressing a CRE-recombinase, flunarizine serum levels reached
an average level of 133nM, which is close to the *in vitro* EC$_{50}$ value described above. Importantly, at this dose HCV-CRE-dependent activation of luciferase expression was significantly repressed (Fig. 1D). Thus, flunarizine inhibits HCV cell entry into human primary liver cells both *in vitro* and humanized mouse hepatocytes *in vivo*.

Since flunarizine did not inhibit infection by vesicular stomatitis virus (VSV) or human coronavirus (Supporting Figure S4), we investigated if it prevents HCV entry across all seven HCV genotypes. Therefore, Huh7-Lunet/hCD81/G-Luc cells were inoculated with chimeric HCV viruses in the presence of increasing doses of flunarizine (Fig. 2A). All non-GT2 strains displayed resistance towards this drug with IC$_{50}$ values more than an order of magnitude greater than Jc1. The GT2b isolate showed an intermediate phenotype with an IC$_{50}$ value of 3.69µM and both GT2a isolates -JFH1 and Jc1- were highly susceptible displaying IC$_{50}$ values of 0.37µM and 0.22 µM, respectively. To map the determinants of flunarizine susceptibility, we shuffled viral core to NS2 proteins between the resistant Con1 (GT1b) and the susceptible Jc1 (GT2a). Transfer of Con1-derived p7 into Jc1 did not render the resulting virus (Jc1/Con1-p7) resistant to flunarizine (Fig. 2B). In contrast, insertion of the Con1-derived E1-E2 proteins into Jc1 did confer flunarizine resistance to Jc1/Con1-E1-E2. Conversely, replacement of the Con1-derived E1-E2 sequences by those of Jc1 rendered the Con1 chimera (Con1/-J6-E1-E2) susceptible to the drug (Fig. 2B). Therefore, susceptibility to flunarizine is governed by determinants resident in the E1-E2 genes. Likewise, when we exchanged structural proteins, p7 or NS2 between J6 (GT2a) or J8 (GT2b), all viruses carrying the J6 E1-E2 genes displayed greater susceptibility to flunarizine compared with those harboring the J8-derived glycoproteins (Supporting Figure S5A). A chimeric virus carrying J8 derived E2 in the backbone of Jc1 produced infectious virus and exhibited partial resistance to flunarizine indicating that determinants in both E1 and E2 influence susceptibility to this drug (Supporting Figure S5B). Next, we used HCV particles trans-
complemented with primary E1-E2 gene sequences from GT2a- and GT2b- infected patients (13). Also these clinical GT2a isolates were susceptible to flunarizine, whereas the GT2b isolates were resistant (Fig. 2C). Finally, we created HCV trans-complemented particles carrying E1-E2 proteins from six additional GT2 subtypes including d, e, k, m, q, and r. All of these strains were susceptible to inhibition by flunarizine, albeit to variable degrees (Fig. 2D). Collectively, this indicates that flunarizine interferes with HCV entry by targeting viral E1-E2 protein function(s) of almost all GT2 subtypes tested and with a clear preference for GT2 over other viral genotypes.

Flunarizine resistance maps to both E1 and E2 and increases susceptibility to cross-neutralizing antibodies. To corroborate that E1 and E2 are targeted by flunarizine, we passaged HCV in presence of flunarizine. After ten weeks we observed drug resistance as infection of Huh7-Lunet/hCD81/G-Luc cells by virus populations cultured in the presence of the drug was poorly inhibited by flunarizine compared to viruses cultured in the presence of DMSO (Fig. 3A). Sequencing revealed conserved amino acid mutations at positions **M267V in E1 (A1140G in H77 genome), Q289H in E1 (A1208C in H77 genome), M405T in E2 (T1554C in H77 genome) and I757T in p7 (T2603C in H77 genome)**, which we engineered into the parental Jc1-luciferase virus either alone or in combination. Insertion of the p7 mutation did not change susceptibility to flunarizine (Fig. 3B). In contrast, the E2 mutation and both mutations in E1 increased virus resistance. A combination of all three envelope protein mutations conferred maximal resistance (ca. 50-fold change in IC_{50}).

Interestingly, E1 residue **Q98-Q289** is fully conserved among all HCV sequences deposited in the gene bank database (Fig. 3C). Additionally, 88.5 % of all GT2 isolates carry **M76-M267** which correlates with susceptibility to flunarizine in our GT2a virus Je1, whereas all non-GT2 sequences encode glycine at this position (Supporting Table ST3). Finally, the E2
mutation resides in the hypervariable region, an important yet strain specific viral neutralizing epitope. The full conservation of 98-289 in E1 and the high level of conservation of 76-267 in E1 may indicate important functional constraints that could limit viral escape in vivo. To explore this, we evaluated neutralization of the flunarizine resistant virus by potent monoclonal antibodies. Neutralization by the E2-targeting HC-11 and AR4A antibodies was significantly enhanced by the flunarizine resistance mutations (Fig. 3D). Collectively, a combination of three mutations – in part affecting highly conserved residues– is necessary to confer a 50-fold resistance to flunarizine. These mutations render HCV more susceptible to neutralizing antibodies, suggesting a high barrier to viral resistance.

Flunarizine inhibits HCV membrane fusion. To precisely define the mode of action of flunarizine, we conducted time-of-addition experiments including HCV entry inhibitors which arrest HCV infection at distinct stages of cell entry (Supporting Figure S6). Since flunarizine resistance was reached at a time point similar to resistance to inhibitors of endosomal acidification (e.g. concanamycin A), this suggested that flunarizine inhibits a late entry step. To explore this further, we used an HCV plasma membrane fusion assay where cells are pre-treated with concanamycin A to prevent viral entry and membrane fusion via the normal route through acidified endosomes (7) (Fig. 4). Under these circumstances, HCV fusion and thus productive infection can be triggered by briefly exposing virus inoculated cells to a low pH buffer. Because HCV membrane fusion requires receptor interactions including the binding to CD81 (14) the virus is receptive to this exogenous trigger (low pH buffer) only after an incubation of ca. 1h at 37°C (7). Thus, Huh7-Lunet/hCD81 cells were continuously treated with concanamycin A and additional drugs were added either directly after virus inoculation and throughout the experiment until 4h post temperature shift (Fig. 4A, protocol I), only during the incubation with low pH
buffer (protocol II) or only directly subsequent to the fusion triggering low pH wash (protocol III). A buffer with neutral pH 7 was used to control that productive infection fully depends on low pH-induced fusion. As expected, HCV infection only occurred when cells were exposed to low pH buffer, since only then luciferase expression above the background of uninfected cells was detected (Fig. 4B). When DMSO was added to the cells according to protocol I, II or III, the low pH wash resulted in high luciferase activity approximately 50-fold above the background of mock infected cells or virus inoculated cells treated with the neutral pH buffer (Fig. 4B). Addition of bafilomycin A1, which like concanamycin A prevents acidification of endosomes, did not have an antiviral effect, regardless of the administration protocol. This confirms that HCV can only access cells by exogenous administration of low pH, independently of endosomal acidification. Moreover, all inhibitors had completely lost antiviral activity when applied directly after the low pH fusion trigger, indicating that they exert their antiviral activity by blocking HCV entry during or upstream of membrane fusion (Fig. 4B, protocol III). Both BJ486K and flunarizine reduced HCV infection essentially to background levels when administered directly after virus inoculation, and thus when present during virus trafficking at the cell surface (1h 37°C) and the low pH-fusion treatment. However, only flunarizine was fully antiviral when added selectively during the five minutes low pH washing step (Fig. 4B, protocol II). Interestingly, viruses carrying the two E1 resistance mutations were no longer inhibited (Fig. 5C), whereas the E2 mutation alone was not sufficient to confer flunarizine resistance in this fusion assay. These results indicate that flunarizine specifically targets HCV membrane fusion and that resistance is primarily mediated by two mutations within E1.

To further corroborate that flunarizine targets HCV fusion, we utilized an imaging assay of fluorescent DiD-HCV entry into polarized, three dimensional hepatocyte cultures (Baktash et al., unpublished data), which enables quantification of HCV single particle cell surface trafficking to
the tight junction complex, virus internalization into early endosomes and ultimately fusion and uncoating. Following DiD-HCV fusion and uncoating, there is an increase in distribution of fluorescence due to mixing of DiD with cellular membranes, which can be quantified (15). Huh-7.5 cells were grown in extracellular matrix to form hepatic spheroids, were treated with either flunarizine or the vehicle control, infected with DiD-HCV and imaged for colocalization with either the tight junction marker zona occludins (ZO)-1 or the early endosomal marker early endosomal antigen (EEA)-1 over a time course of entry. We observed that flunarizine did not affect the localization of DiD-HCV to the tight junction (Fig. 5A, B) nor did it affect the internalization and localization of DiD-HCV with the early endosome (Fig. 5C, D). Treatment with flunarizine did decrease DiD-HCV fusion, which is evidenced by significantly lower DiD fluorescence volume at 360min post temperature shift as compared to the vehicle control (Fig. 5E,F). Therefore, flunarizine does not affect HCV trafficking and endocytosis but inhibits fusion of HCV with the endosomal membrane.

**Phenothiazines and Diphenylmethylpiperidines but not Ca\(^{2+}\) channel inhibitors in general prevent HCV fusion in a genotype-dependent manner.** Flunarizine is a T-type Ca\(^{2+}\) channel inhibitor and belongs to the group of diarylmethyl piperazine drugs. A range of structurally related drugs (e.g. pimozide) are known which also block Ca\(^{2+}\) ion channels and which are used as anti-psychotic drugs (16). Moreover, phenothiazines such as fluphenazine and trifluoperazine are also important drugs in which the phenothiazine group resembles the biarylmethyl group. Indeed, all of these have emerged as potent HCV inhibitors in our screening (Supporting Figure S1). Therefore, we explored if inhibition of Ca\(^{2+}\) ion channels in general is essential for the antiviral activity of these molecules. Furthermore, we investigated if these drugs inhibit HCV via an antiviral mechanism comparable to flunarizine. To address this we explored the influence of...
1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA) and ethylene glycol tetraacetic acid (EGTA), two Ca\(^{2+}\)-chelating agents, which sequester intracellular and extracellular Ca\(^{2+}\)-pools, respectively, on the antiviral activity of flunarizine. Although both BAPTA and EGTA were administered at high doses, they did not modulate the antiviral activity of flunarizine indicating inhibition of HCV entry by flunarizine does not depend on availability of Ca\(^{2+}\) (Fig. 6A). Next we explored if mibefradil (17), penfluridol (18), or NiCl\(_2\) (19), three well established inhibitors of T-type Ca\(^{2+}\) channels, interfere with HCV infection. Neither of these molecules inhibited infection of parental HCV or flunarizine-resistant HCV (Fig. 6B). In contrast, both phenothiazines (fluphenazine and trifluoperazine) and pimozide preferentially inhibited the parental virus and were less active against the variant with flunarizin resistance mutations. The antiviral activity of BJ486K (20) and curcumin (21) - two molecules that inhibit HCV cell entry by different molecular mechanisms - was not influenced by these mutations (Fig. 6B). While this work was in preparation, Chamoun-Emmanueli reported that selected phenothiazines inhibit HCV entry potentially by increasing the fluidity of cholesterol-rich membranes (22). This prompted us to explore if viral susceptibility to flunarizine correlates with susceptibility to modulation of membrane fluidity achieved by depletion of cholesterol using methyl-beta-cyclodextrin. Interestingly, we observed that Jc1 was significantly more susceptible to cholesterol depletion than a genotype 5a virus, which has high endogenous flunarizine resistance (Fig. 6C). Moreover, also the flunarizine-resistant Jc1-variant was significantly more resistant to depletion of cholesterol compared with parental Jc1 (Fig. 6C). This suggests that differences in the dependence of the HCV fusion machinery on membrane properties may in part determine susceptibility to flunarizine, phenothiazines and pimozide. However, in our experiments these drugs did not modulate membrane fluidity (Supporting Figure S7) nor cellular cholesterol content.
(Supporting Figure S8), suggesting that other membrane changes are responsible for fusion inhibition.

Notably, pimozide was antiviral \textit{in vivo} (Fig. 1D), and the individual mutations in E1 and E2 conferred partial resistance to pimozide like they did to flunarizine (Supporting Figure S7S9). Finally, fluphenazine, trifluoperazine and pimozide displayed a preference for GT2a over the other GTs, very similar to flunarizine. However, pimozide and fluphenazine displayed a much improved cross-genotype coverage with IC50 values below 10\(\mu\)M for GT 3a, 5a and 7a in case of pimozide and for GT3a, 6a and 7a for fluphenazine (Fig. 7). In conclusion, the antiviral activity of flunarizine and related compounds is not directly linked with their ability to inhibit Ca\(^{2+}\) ion channels. Nevertheless, viral cross-resistance and comparable genotype-specificity between flunarizine, these phenothiazines and pimozide, strongly argue for a shared antiviral mode of action.

\section*{Discussion}

We screened a library of clinically licensed drugs and identified four related ion channel inhibitors that prevent HCV entry \textit{in vitro} and \textit{in vivo}. We provide evidence based on chimeric viruses and resistance mutations that these molecules selectively target HCV E1-E2 functioning during entry. Moreover, time of addition experiments and single particle tracking indicate that these drugs specifically inhibit HCV membrane fusion. Finally, the domain of E1 carrying the resistance mutations likely plays a key role in membrane fusion.

Several scenarios how these molecules prevent HCV membrane fusion are possible. Firstly, these drugs which all inhibit cellular Ca\(^{2+}\)-channels, may prevent fusion by changing Ca\(^{2+}\)-fluxes in HCV target cells and/or by directly blocking the interaction of HCV with a Ca\(^{2+}\)-channel critical for membrane fusion. However, not all tested Ca\(^{2+}\)-channel targeting drugs also
arrested HCV infection. Moreover, sequestration of Ca\(^{2+}\) did not modulate the antiviral activity of flunarizine. Therefore, we consider it unlikely that inhibition of Ca\(^{2+}\)-channels by these drugs - which then would be used by HCV in a genotype-dependent fashion - is responsible for the anti-HCV activity of this class of molecules.

Secondly, it is possible that flunarizine, pimozide and phenothiazines directly bind E1-E2, thereby inhibiting membrane fusion. The genotype-specificity of the antiviral activity supports this notion since sequence variation may prevent drug binding. However, HCVpp carrying the same viral envelope proteins as flunarizine-susceptible HCVcc particles are not inhibited by flunarizine, arguing against this. Also Chockalingam et al. and Hu et al. reported resistance of HCVpp to flunarizine leading them to conclude that flunarizine is likely not an entry inhibitor (10,11). Our data, however, show that flunarizine inhibits entry of authentic HCV at the stage of membrane fusion. This highlights an as yet unexplored difference between the mode of HCVpp and HCVcc membrane fusion which may arise because HCVpp are produced in 293T cells rather than in human liver cells. Consequently, HCVpp could differ in lipid and lipoprotein composition thus affecting cell entry. Notably, it has been reported that receptor usage can differ between HCVcc and HCVpp with only the former using the Niemann-Pick C1-like 1 cholesterol absorption receptor for cell entry (23). Furthermore, Meertens and colleagues observed that cholesterol depletion of the host cell had no effect on the entry of HCVpp, whereas it was antiviral for HCVcc (24). Therefore, the resistance mutations identified by us could be part of a direct drug binding site, or they could modulate the conformation and function of E1-E2 in membrane fusion which seems to be exquisitely cholesterol dependent for HCVcc particles. The E1 domain from residue 73-264 to 99-290 has previously been implicated in harboring an HCV fusion peptide based on phylogenetic comparison with related flaviviruses, sequence conservation and hydrophobicity (25). The observation that drugs that specifically inhibit
membrane fusion select for viral resistance in this region indirectly supports the notion that this peptide is critical for fusion. The crystal structures of E2 did not reveal evidence for a major role of E2 as fusion protein so that E1 is now assumed to be the primary fusion protein of HCV (26,27). This is also supported by earlier studies (28-30).

Finally, it is possible that these lipophilic drugs perturb membrane properties critical for virus membrane fusion. In fact, while this work was in preparation Chamoun-Emanuelli reported that this was the case for specific phenothiazines including fluphenazine and trifluoperazine, also characterized by us (22). Intriguingly, we observed a correlation between HCV susceptibility to flunarizine and susceptibility of HCV to cholesterol-depletion induced changes in membrane properties. This result is compatible with the notion that also flunarizine and pimozide – like phenothiazines – modulate membrane fluidity and that this is the antiviral principle of these groups of compounds. In turn, the genotype-specific antiviral activity of these molecules implies that viral strains differ with regard to membrane requirements during fusion. The cholesterol depletion experiment reported here, which shows that Jc1 is more susceptible to cholesterol depletion compared to the flunarizine resistant Jc1 and to a flunarizine resistant strain (GT5a) supports this notion. However, unlike Chamoun-Emanuelli but consistent with Thomas and Verkleij we did not observe a modification of membrane fluidity by flunarizin and related drugs (31). This discrepancy between the Chamoun-Emanuelli studies may be due to differences in the assay set up including the composition of liposomes (Supporting Figure S7). Moreover, flunarizine and related compounds did not extract cholesterol from Huh-7.5 cells - even upon prolonged incubation (Supporting Figure S8).

Flunarizine also affects other biophysical membrane properties. For example, it inhibits the formation of hexagonal HII phase (31), which is homologous to the formation of the negative curvature required for fusion of viral and cellular membranes (32,33). Notably, cholesterol
depletion also affects the ability of a lipid bilayer to form the negative curvature required for fusion. Flunarizine may therefore inhibit HCV infectivity by affecting biophysical properties of the cellular membranes which are also dependent on cholesterol content, other than fluidity.

It will be interesting to explore viral determinants responsible for differential cholesterol-dependence between viral strains and how these differences relate to viral polymorphisms within the putative fusion peptide (residues 73 to 99 of E1).

A combination of three amino acid changes was necessary to obtain a 50-fold viral resistance to flunarizine. Moreover, the resistance mutation affected in part completely or highly conserved residues and significantly increased virus neutralization by two cross-neutralizing antibodies suggesting that the barrier to viral resistance could be relatively high in vivo. Therefore, flunarizine and related compounds merit consideration for repurposing as potential adjunct therapy for GT2 infected patients and due to broader GT coverage in case of pimozide, trifluoperazine and fluphenazine also for the difficult to treat GT3.

Acknowledgments:

We thank Gabriela Hrebikova for outstanding technical assistance, all members of the Institute of Experimental Virology and Mark Brönstrup, Beate Sodeik, and Stephan Pöhlmann for critical discussion of this work. We are grateful to Takaji Wakita for the gift of the JFH1 isolate, to Jens Bukh for the J6 strain and to Charles Rice for Huh-7.5 cells and 9E10 antibody. We also would like to thank Steven Foung, Arvind Patel and Mansun Law for providing anti-E2 monoclonal antibodies, and Volker Thiel and Gert Zimmer for sharing Corona- and VSV-reporter viruses.

References


33. Colpitts CC, Ustinov AV, Epand RF, Epand RM, Korshun VA, Schang LM. 5-(Perylen-3-yl)ethynyl-arabino-uridine (aUY11), an arabino-based rigid amphipathic fusion inhibitor, targets virion envelope lipids to inhibit fusion of influenza virus, hepatitis C virus, and other enveloped viruses. *Journal of virology*. 2013;87(7):3640-3654.

**Figure legends**

**Fig. 1.** Flunarizine inhibits HCV entry into human hepatocytes *in vitro* and *in vivo*. (A) Huh7-Lunet/hCD81/G-Luc cells expressing gaussia luciferase were transfected with F-Luc-Jc1. After 4h, medium with 2-fold dilutions of flunarizine was added. Measurements of G-Luc (cell viability) and F-Luc (RNA replication) were taken after 48h. Viruses produced at this time point were used to inoculate target cells where F-Luc activity was determined 48h later (whole life cycle). (B) Huh7-Lunet/hCD81/G-Luc cells were inoculated with F-Luc-Jc1 and flunarizine or solvent for 4h. Infection was measured 48h afterwards (C) Primary human hepatocytes were incubated with the indicated compounds and Jc1 for 6h. The supernatant of inoculated cells was collected at 24 and 48h and virus infectivity was determined by TCID<sub>50</sub>. (D) HCV entry reporter mice expressing firefly luciferase in a Cre-dependent manner were pretreated with indicated compounds. Subsequently, they were challenged with a Jc1 variant expressing Cre recombinase.
HCV-Cre-dependent luciferase expression, which reflects HCV cell entry efficiency, was
determined 24h later.

**Fig. 2.** The antiviral activity of flunarizine is HCV strain-dependent and viral determinants
governing susceptibility reside within the E1-E2 genes. (A) Chimeric renilla luciferase reporter
viruses (left panel) or F-Luc-Jc1 or F-Luc-JFH-1 (right panel) were inoculated with 2-fold
dilutions of flunarizine into Huh7-Lunet/hCD81/G-Luc cells for 4h. Luciferase levels were
measured 48h post-infection. (B) Chimeras between flunarizine susceptible Jc1 (GT2a) and
flunarizine resistant Con1/C3 (GT1b) were created as indicated and tested for their susceptibility
to flunarizine by using a focus formation unit assay. (C) HCV trans-complemented particles
harboring GT2a or GT2b E1E2 glycoproteins of given primary, patient-derived viruses (13) were
used to inoculate cells in the presence (2-fold dilutions) or absence of flunarizine for 4h. Infection
efficiency was determined 48h post inoculation using renilla luciferase assays. (D) HCV trans-
complemented particles harboring E1E2 glycoproteins of representatives of indicated GT2
subtypes (2) were used to inoculate cells in the presence of (2-fold dilutions) or absence of
flunarizine. Infection efficiency was determined 48h post inoculation by focus formation unit
assay.

**Fig. 3.** Two mutations of conserved residues in E1 and one mutation in E2 confer resistance to
flunarizine. (A) Jc1 was passaged in Huh7-Lunet/hCD81/G-Luc cells in the presence of given
doses of flunarizine or in the presence of DMSO. The resulting virus populations were used to
inoculate naïve Huh7-Lunet/hCD81/G-Luc cells for 4h in the presence of 5.25 or 10.5µM of
flunarizine or DMSO. The infection was assessed 48h later by immune staining of the NS5A
protein (green). DNA in the nucleus was counterstained with DAPI (blue). (B) Jc1 luciferase reporter virus (WT) or derivatives thereof carrying the indicated point mutations were used to infect Huh7-Lunet/hCD81/G-Luc cells in the presence of 0, 0.65, 1.3, 2.6, 5.25, 10.5µM of flunarizine for 4h. Renilla luciferase activity was measured 48h later. (C) Prevalence of indicated amino acids at positions 76-267 or 98-289 in HCV E1 across all GT1-GT7 strains deposited in the HCV database. The total number of analyzed sequences for each genotype is stated in brackets. (D) Parental or flunarizine-resistant Jc1 was incubated for 1h at 37°C with serial dilutions of given monoclonal antibodies targeting E2 (AP33, CBH23, or HC11) or a discontinuous epitope of the E1-E2 complex (AR4A) before inoculation of Huh7-Lunet/hCD81/G-Luc cells for 4h at 37°C. After 48h, cells were lysed and renilla reporter activity was measured.

Fig. 4. Flunarizine inhibits HCV membrane fusion at the plasma membrane. (A) Schematic representation of the experimental procedure. Huh7-Lunet/hCD81/G-Luc cells were incubated with 5nM Concanamycin A (ConA) 1h before virus inoculation and throughout the experiment until 4h post virus inoculation. Additional drugs or DMSO were applied as indicated by black bars according to protocols denominated I, II or III. F-Luc Jc1 particles were inoculated for 2h at 4°C. Virus membrane fusion at the plasma membrane was triggered by washing cells with a pH5 buffer (or a pH7 buffer as control) for 5 min 1h after inoculated cells were shifted to 37°C. In all treatments, cells were incubated another 48h at 37°C before infection efficiency was quantified by luciferase assays. (B) F-Luc-Jc1 dependent luciferase expression in cells inoculated according to protocol (I), (II) or (III) and with pH5 or pH7-buffer treatment. (C) R-Luc-Jc1-dependent luciferase expression in cells inoculated with parental Jc1 (WT) or with Jc1-derivatives carrying indicated resistance mutations. Infection was conducted according to protocol II depicted in panel (A).
Fig. 5. Treatment with flunarizine perturbs DiD-HCV endosomal membrane fusion. Huh-7.5 organoids were incubated with 10uM flunarizine or 10% DMEM alone (VC) for 1h, infected with DiD-HCV for 1h at 4°C, shifted to 37°C for the indicated times, fixed, and probed for tight junction protein ZO-1 (A) or early endosomal marker EEA1 (C). (A) Tight junction region is shown in the inset. Left: ZO-1 (green), Right: DiD-HCV (red). (B) Quantitation of (A). (C) Arrows indicate DiD-HCV particles enlarged in insets. Left: EEA1 (green), Right: DiD-HCV (red). DiD-HCV colocalization with EEA1 antibody is indicated by dashed arrow. (D) Quantitation of (C). (E) DiD fluorescence over EEA1 time course. (F) Average DiD fluorescence per cluster in (E). n=total DiD signal (B, D) or total clusters (E); mean +/- SD. **p<0.001. VC = vehicle control.

Fig. 6. Flunarizine-resistant HCV is cross-resistant to phenothiazines and pimozide, antiviral activity is not modulated by Ca\(^{2+}\) sequestration. (A) Huh7-Lunet/hCD81/G-Luc cells were infected with F-Luc-Jc1 in presence of increasing concentrations of flunarizine either alone or together with the intracellular calcium chelator BAPTA-AM or the extracellular calcium chelator EGTA. After 2h, the viruses and compounds were washed away with PBS twice and fresh medium was added. The cells were lysed and the firefly levels measured 48h post-infection. (B) Huh7-Lunet/hCD81/G-Luc cells were infected with parental Jc1 renilla reporter viruses (WT) or with the flunarizine-resistant Jc1 variant (M267V/ Q289H/ M405T/ I757TE1 M76V/E1 Q98H/E2 M22T/p7 I7P) for 4 h in presence of serially diluted mibebradil, penfluridol or NiCl\(_2\). Medium was changed and 48h later cells were lysed and measured for renilla luciferase activity. (C) Huh7-Lunet/hCD81/G-Luc cells were pre-treated for 1h with increasing concentrations of...
methyl-β-cyclodextrin to deplete membrane cholesterol. After washing with PBS, cells were
inoculated with given renilla reporter viruses for 4h. The cells were lysed and the renilla levels
measured 48h post-inoculation.

Fig. 7. Fluphenazine, pimozide and trifluoperazine preferentially inhibit HCV infection in a
genotype-dependent fashion, but are more active against other genotypes than flunarizine. (A)
Huh7-Lunet/hCD81/G-Luc cells were inoculated with renilla luciferase reporter virus chimeras
of GT2a (Jc1), 3a (S52), 5a (SA13), 6a (HK6a) or 7a (QC69) together with 2-fold dilutions of
flunarizine, fluphenazine, pimozide or trifluoperazine. After 48h cells were lysed and measured
for renilla luciferase activity. Means and standard deviations of 3 independent experiments are
given. (B) The calculated IC$_{50}$ of each compound against each indicated genotype represented in
panel (A) was plotted for comparison.
Figure 1
188x162mm (300 x 300 DPI)
Figure 2

(A) R-luc reporter viruses

(B) F-luc reporter viruses

189x275mm (300 x 300 DPI)
Figure 3

(A) DMSO, Flunazine (5.25 μM), and Flunazine (10.5 μM) treatments on DMSO-resistant cells.

(B) Graph showing % infection with different concentrations of Flunazine (0.14 μM to 10.5 μM).

(C) Comparison of M207V and Q200I mutations in terms of % enzymes in position.

(D) Effects of APV33, HC11, ARA, and CBII23 on % infection.

Figure 3
203x281mm (300 x 300 DPI)
Figure 4

(A) Diagram showing the experimental protocol for measuring the effect of Flunarizine addition on virus infection.

(B) Graph showing the infection rate (RLU) for different conditions:
- Pre-pH shift addition
- pH shift addition (5 min)
- Post-pH shift addition

(C) Comparison of infection rates (RLU) between pH 7.0 and pH 5.0 for different strains:
- WT
- M267V/Q289H
- M405T/1757T
- M267V/Q289H (E1/E1)
- M405T (E2)

Figure 4
197x210mm (300 x 300 DPI)
Figure 5

188x242mm (300 x 300 DPI)
Figure 6

(A) Flunarizine, Flunarizine + BAPTA (12.5 μM), Flunarizine + EGTA (2 μM)

(B) Mifepristone (μM), Penfluridol (μM), NiCl₂ (μM)

(C) Jc1 (M267V/ Q289H/ M405T/ I757T), Jc WT

WT, M267V/ Q289H/ M405T/ I757T

Fluphenazine (μM), Pimozide (μM), Trifluoperazine (μM)

*** p < 0.001

Figure 6
202x246mm (300 x 300 DPI)
Figure 7

(A) 

(B) 

149x211mm (300 x 300 DPI)
Supporting Information

Supporting materials and methods

Cell culture: Huh-7.5 (1), Huh7-Lunet/hCD81 expressing firefly (Fluc) or gaussia luciferase (Gluc) (2,3), the packaging cell line Huh-7.5[Cl[p7NS2]J6 (4) and 293T (DuBridge) cells were cultured in Dulbecco’s modified Eagle’s medium (Life Technologies) supplemented with 2mM L-glutamine (Life Technologies), non-essential amino acids (Life Technologies), 100U of penicillin (Life Technologies) per ml, 100μg of streptomycin (Life Technologies) per ml, and 10% fetal calf serum (PAA Laboratories GmbH) at 37°C and 5% CO2. If required, blasticidin (5 μg/mL) was added for selection of transduced cells. Primary human hepatocytes were cultured in HCM (Lonza; HBM with single quots of ascorbic acid, hydrocortisone, transferrin, insulin, gentamycin, BSA and rhEGF) supplemented with 10% human serum (PAA Laboratories GmbH). For three-dimensional hepatocytes cultures, Huh-7.5 cells were trypsinized and diluted in DMEM + 10% FBS to a final concentration of 1 x10^5 cells/mL. Equal volumes of thawed Matrigel (Growth Factor Reduced, Phenol Red-free; BD Biosciences) and diluted cells were combined, and then seeded onto coverslips in 24-well plates (75 μL/well). The cell-Matrigel solution was allowed to polymerize for 30 minutes at 37°C before adding DMEM + 10% FBS. Cells were grown for 7 days, changing media every other day. For three-dimensional hepatocytes cultures, Huh-7.5 cells were trypsinized and diluted in DMEM + 10% FBS to a final concentration of 1 x10^5 cells/mL. Equal volumes of thawed Matrigel (Growth Factor Reduced, Phenol Red-free; BD Biosciences) and diluted cells were combined, then seeded onto coverslips in 24-well plates (75 μL/well). The cell-Matrigel solution was allowed to polymerize for 30 minutes at 37°C before adding DMEM + 10% FBS. Cells were grown for 7 days, changing media every other day.

Plasmids: The full length JFH-1 with firefly luciferase (5); The chimeric 2a/2a Jc1 virus, with (pFK-Luc-Jc1) or without (pFK-Jc1) firefly luciferase (5,6); The patient-derived ΔCE1E2 genes
into the replicon pFK PI-E1-NS3-NS5B/JFH1 (4); The Jc1-derived virus expressing a renilla luciferase (7); The monocistronic renilla luciferase reporter virus genomes H77c/1a/R2a, J4/1b/R2a, JcR2a, J8/2b/R2a, S52/3a/R2a, ED43/4a/R2a, SA13/5a/R2a, HK6a/6a/R2a and QC69/7a/R2a (8); The pFK-Con1/C3 referred herein as Con1 (9), JFH1/Con1/C3/E1E2J6 referred herein as Con1 J6 E1E2 and Jc1 Con1 E1E2 (10); Jc1/p7-Con1 chimeras referred herein as Jc1 Con1 p7 (11); The firefly luciferase Jc1 containing the E2 mutation G451R (8) and the Jc1 lacking the hypervariable region 1 (Jc1 ∆HVR1) (12) have been previously described elsewhere. Cloning strategies for the construction of the renilla reporter chimeric viruses between J6 (2a) and J8 (2b), the flunarizine-resistant mutants and the Jc1 containing a mutation in E1 (E1 M76G) can be obtained upon request.

**Time-of-addition assay:** Time-of-addition experiments were performed similarly to what was previously published (8). Huh7-Lunet/hCD81 cells seeded one day before in 12-well plates (5.3 x 10^4 cells/well) and infected with firefly reporter Jc1 viruses at 4°C for 1 h, so that infections were synchronized. Cells are washed twice with PBS and fresh medium is added before the cells are transferred at 37°C, so that infection can proceed. Heparin (50 µg/mL), JS81 antibody (2 µg/mL) or Concamycin A (5 nM) were added at different time points in each of the eight simultaneously performed protocols. Either the compounds were added during binding at 4°C or after transfer to 37°C (20, 40, 60, 80, 100, 120, 180 minutes after transfer to 37°C). Incubation with the compounds in each protocol was always of 4 h, followed by media change. After 48 h, cells were washed, lysed and luciferase activity was measured.

**Primary human hepatocytes:** Primary human hepatocytes were isolated applying a 2-step collagenase perfusion technique as previously described (13). Briefly, the liver specimens were flushed once with 500 ml washing buffer containing 2.5 mM EGTA followed by perfusion with 100 ml digestion buffer containing 0.05% collagenase P (Roche Diagnostics) and allowing for
recirculation of the perfusate. Upon sufficient digestion, the tissue was mechanically disrupted, poured through a gauze-lined funnel and centrifuged (50 g, 5 min., 4°C). The resulting cell pellet was washed twice using PBS (50 g, 5 min., 4°C) and resuspended in supplemented William’s medium E (all Biochrom AG, Berlin, Germany) (1 µM insulin, 1 µM dexamethason/fortecortin, 100 U/ml penicillin, 100 µg/ml streptomycin, 1 mM sodium pyruvate, 15 mM HEPES buffer, 4 mM L-glutamine and 5% FCS). Viability was determined by the Trypan blue exclusion test and hepatocytes were seeded in collagen pre-coated 6-well plates at a concentration of 1.5 x 10^6 viable cells/well. Dead and non-adherent cells were removed by medium change 16 to 18 h after plating. The protocol was approved by the local ethics commission, Ethikkommission, Medizinische Hochschule Hannover (# 252-2008). No donor organs were obtained from executed prisoners or other institutionalized persons.

**In vitro transcription and electroporation:** In vitro transcription and electroporation of cells were generated as described previously (5). Linearized plasmid DNA extracted with phenol and chloroform were in vitro transcribed and RNA was extracted with phenol and chloroform. Cells counted to a final concentration of 1 x 10^7 cells/ml were resuspended in Cytomix containing 2 mM ATP and 5 mM glutathione and transfected with respective RNA by electroporation. Transfected cells were immediately transferred to complete DMEM before seeding to dishes.

**Luciferase infection assay:** Huh7-Lunet/hCD81 cells seeded one day before in 12-well plates (5.3 x 10^4 cells/well) were infected with Jc1 with firefly or renilla luciferase for 4 h in presence of different concentrations of compounds. Viruses and compounds were removed, cells were washed with PBS and fresh medium was added. After 48 h, cells were washed with PBS and for firefly viruses, lysed in 350 µL lysis buffer (0.1% Triton X-100, 25 mM glycylglycine, 15 mM MgSO_4_, 4 mM EGTA, 1 mM DTT, pH 7.8) or, for renilla viruses, in 200 µL Passive lysis buffer (Promega) diluted 1:5 in water. Firefly luciferase activity was evaluated by adding 100 µL of
cell lysates into assay buffer (25 mM glycyglycine, 15 mM MgSO$_4$, 4 mM EGTA, 1 mM DTT, 2 mM ATP, 15 mM K$_2$PO$_4$) and luciferin solution (200 µM luciferin, 25 mM glycyglycine, pH 8) and measured for 20 seconds in a luminometer (Lumat LB9507; Berthold). Renilla luciferase activity was measured by adding 20 µL of cell lysates into renilla luciferase substrate in PBS (1 µM of coelenterazin; P.J.K.) and measured for 1 second in a luminometer (Lumat LB9507; Berthold).

**Virus titration by immunohistochemical staining:** Virus titration by this limiting dilution assay was determined according to a previously established protocol (14). Briefly, Huh7.5 cells were seeded into 96-well plates at a concentration of 1 x 10$^5$ cells/mL. One day later, these cells were infected with serial dilutions (1:5) of filtered virus stocks. Three days later, cells were washed with PBS, fixed for 20 min with ice-cold methanol at -20°C and washed three times with PBS. The viral protein NS5A was detected with a 1:1000 dilution of mAb 9E10 (15) in PBS for 1 h at room temperature or overnight at 4°C. Cells were washed three times with PBS, and a peroxidase-conjugated antibody specific to murine IgG (Sigma-Aldrich) diluted at 1:200 in PBS was added to each well. Following 1 h incubation at room temperature, peroxidase activity was detected by incubating cells with 0.32% (w/v) of 3-amino-9-ethylcarbazole (Sigma) in N,N-dimethylformamide diluted at a ratio of 1:3.3 with 15 mM acetic acid, 35 mM sodium acetate, pH 8.0, and 0.4% H$_2$O$_2$. Cells were incubated for 10-30 minutes at room temperature, the carbazole substrate was removed and water was added. The 50% tissue culture dose was calculated by the method of Spearman and Kärber.

**Indirect immunofluorescence:** Cells were fixed with 3% paraformaldehyde in PBS for 5 minutes. The NS5A protein was detected by incubation with mouse monoclonal E9E10 antibody diluted 1:2000 in PBS and supplemented with 5% goat serum (Sigma), for 1 h at room temperature. Cells were washed with PBS and a secondary antibody specific for murine IgG
conjugated with Alexa-Fluor 488 (Invitrogen) at a dilution of 1:1000 was added to the cells for 1 h at room temperature. Cell nuclei were counter-stained for 1 minute at room temperature with DAPI (Invitrogen) diluted 1:3000.

**Matrigel polarization, DiD-HCV preparation and imaging**

Huh-7.5 cells were trypsinized and diluted in DMEM + 10% FBS to a final concentration of 1 x10⁵ cells/mL. Equal volumes of thawed Matrigel (Growth Factor Reduced, Phenol Red-free; BD Biosciences) and diluted cells were combined and seeded onto coverslips in 24-well plates (75 µL/well). The cell-Matrigel solution was allowed to polymerize for 30 minutes at 37°C before adding DMEM + 10% FBS. Cells were grown for 7 days, changing media every other day.

Viral stocks were concentrated via PEG (polyethylene glycol 8000; Fisher) precipitation (Blight et al., 2002; Coller et al., 2009), then resuspended in serum free media. One mL of concentrated virus was incubated with 5 µL of DiD (Invitrogen) for 90 minutes. Labeled virus was layered onto a 10–60% weight/volume iodixanol gradient (OptiPrep, Sigma) and centrifuged for 16.5 hours (34,000 RPMs at 4°C). The gradient was then fractionated; each 1 mL fraction was subsequently analyzed for specific infectivity (HCV RNA levels via Trizol-LS extraction, Invitrogen, and infectious virus). Fractions with the best specific infectivity (5.7 ± 3.1) were purified with Amicon Ultra 100k filters (Millipore) and pooled for use in imaging studies.

Matrigel-polarized cells were preincubated on ice for 20 minutes. DiD-labeled HCV was added to cells and incubated on ice for an additional hour, then transferred to 37°C (time of temperature shift: t=0) and fixed at various points after the shift in 3.6% paraformaldehyde for 20 minutes. Cells were permeabilized with 0.5% Triton x-100 in PBS for 10 minutes, and then washed three times with 0.1 M Glycine in PBS for 10 minutes each. Cells were incubated for 2 hours in blocking solution (Wash buffer: 0.1% BSA, 0.2% Triton x-100, 0.005% Tween-20 in PBS + 20% goat serum). Coverslips were incubated overnight at 4°C with primary antibodies diluted in...
blocking solution. (1:500 anti-ZO-1, Invitrogen; 1:400 anti-EEA1, Abcam; 1:350 anti-Core, Virostat). Following primary incubation, coverslips were washed 3 times for 20 minutes with wash buffer. Alexa Fluor conjugated secondary antibody (488 or 594) was diluted 1:1000 in blocking solution and incubated for 1 hour at room temperature, rinsed 3 times with wash buffer (as above), then mounted with DAPI prolong gold (Invitrogen).

Imaging was performed on an Olympus DSU Spinning Disc Confocal with an 100X NA 1.45 oil-immersion objective. Using Slidebook imaging software, images were captured with a Hamamatsu back thinned EM-CCD camera set to an intensification of 255. DiD-labeled HCV and Alexafluor 594 were visualized with the DsRed filter set; Alexafluor 488 was visualized with the EGFP filter set. Z-stacks of the organoids were acquired using slices taken every 0.3 µm.

Images were quantified for colocalization in ImageJ using RGB profiler (Christophe Laumonerie) and colocalization highlighter. Voxels were calculated using Object Counter 3D. Images presented in the figures were duplicated out of the Z-stack, separated into individual channels, adjusted for contrast and smoothed, then reassembled.

**Membrane Fluidity assays.** DOPC (1,2-dioleoyl-sn-glycero-3-phosphocholine) and cholesterol were dissolved in chloroform to 2.7 µmol/ml at a molar ratio of 2.7:0 (100% DOPC) or 1.7:1.0 (37% cholesterol - 63% DOPC). Lipids were dried by evaporating the solvent in a fume hood overnight and then re-hydrated in 1 ml Na₂HPO₄•citric acid buffer (200 mM Na₂HPO₄•citric acid, pH 7.4) by vortexing for 3-5 minutes. Re-hydrated lipids were extruded through a polycarbonate membrane (pore size ≤0.2 µm) using an avanti mini-extruder kit. 1,6-Diphenyl-1,3,5-hexatriene (DPH) was dissolved in tetrahydrofuran at a concentration of 2 mM. Liposomes (100% DOPC or 37% cholesterol - 67% DOPC) were suspended in Na₂HPO₄•citric acid buffer (pH 7.4) and labelled with DPH at a final concentration of 2µM for 5 minutes at 37°C. DPH-labelled liposomes were treated with the indicated concentrations of curcumin, flunarizine.
dihydrochloride, pimozide, trifluoperazine dihydrochloride, fluphenazine hydrochloride, or DMSO vehicle for 10 minutes at 37°C. Treated liposomes were diluted in Na$_2$HPO$_4$•citric acid buffer (pH 7.4) to 2.5 mL and kept at 37°C. DPH fluorescence polarization was evaluated at 350 nm excitation and 450 nm emission wavelengths using a QuantaMaster 40 scanning spectrofluorometer (Photon Technology International, Birmingham, NJ) equipped with a 75-W xenon lamp. Fluorescence polarization ($P$) was calculated according to the following equation: $P = \frac{(I_{VV} - G I_{VH})}{(I_{VV} + G I_{VH})}$, where $I_{VV}$ and $I_{VH}$ are the fluorescence intensities with parallel and perpendicular polarization to the excitation beam, respectively, and $G$ is the sensitivity of the fluorimeter detector for vertically relative to horizontally polarized light. To test cholesterol dependence, polarization was expressed as a ratio of DPH polarization in liposomes containing 37% cholesterol over the polarization in liposomes with no cholesterol.

**Membrane cholesterol content.** Huh7.5 cells were seeded in 6-well plates at 5 x 10$^5$ cells per well and treated with test compounds or vehicle for 24 h, or with β-cyclodextrin for 3 h, at 37°C. Cells were washed three times with PBS before adding 2 ml of hexane:isopropanol (3:2) and incubating for 1 h on a rocker at RT. Supernatants were transferred to glass tubes and solvent was removed under reduced pressure. Total protein was extracted by addition of 1 mL protein solubilisation buffer (10 mM sodium borate, 1% SDS) after removal of the hexane:isopropanol supernatant. The dried lipids were dissolved in 1 ml cholesterol assay buffer (0.5 M potassium phosphate, pH 7.4, 0.25 M sodium chloride, 25 mM cholic acid, 0.5% Triton X-100) by vortex. Total cholesterol was quantitated in 50 µl of the dissolved lipids using Amplex® Red Cholesterol Assay Kit (Molecular Probes, Inc.) and proteins were quantitated using DC Protein Assay (Bio-Rad). Total cellular cholesterol is presented normalized to protein.
In vivo infection assay: 4EFT Rosa26-LSL-Fluc mice (16) were assigned to three treatment groups. The first group received per timepoint 50 mg/kg bodyweight flunarizine per oral gavage and 50 mg/kg bodyweight intraperitoneally. The second group received per timepoint 50 mg/kg bodyweight flunarizine per oral gavage and 50 mg/kg bodyweight intraperitoneally. A third treatment group only received the vehicles alone. For the oral preparation flunarizine or pimozide respectively were solved in a formulation of HPMC K15 M (Sigma) in a 25mM citrate buffer (pH4.0). For intraperitoneal injections the drugs were dissolved in 0.2 M tartaric acid. The above described doses were administered 21 hours and 2 hours before and 2 hours after infection. Mice were intravenously infected with 2x10^7 TCDID_{50} BiCreJc1. Generation of BiCreJc1 was described elsewhere (17). Rosa26-LSL-Fluc mice not expressing the 4 human entry factors served as negative control. At 48 hours post infection, mice were anaesthetized using isoflurane and injected intraperitoneally with 1.5 mg luciferin (Caliper Lifesciences). Bioluminescence was measured using an IVIS Lumina II platform (Caliper Lifesciences). Concentration of flunarizine and pimozide in murine blood serum was determined as follows. To a 50 µL aliquot of blood serum were added 80 µL of acetone. The suspension was stored at -80°C for 30 min before it was centrifuged. The supernatant was concentrated to dryness and re-dissolved in 30 µL of DMSO. Liquid chromatography-mass spectrometry (LC-MS) measurements were performed on a Thermo Ultimate 3000 RSLC system equipped with a BEH C18 (50×2.1 mm, 1.7µm) column (Waters, Germany). Separation of a 5 µL sample was achieved by a linear gradient from (A) H₂O + 0.1% HCO₂H to (B) CH₃CN + 0.1% HCO₂H at a flow rate of 600 µL/min and 45°C. The gradient was initiated by a 0.5 min isocratic step at 5% B, followed by an increase to 95% B in 9 min to end up with a 1 min step at 95% B before re-equilibration under the initial conditions. UV spectra were recorded by a DAD in the range from 200 to 600 nm. The unsplit LC flow was entering the amaZon speed mass spectrometer (Bruker Daltonics, Germany) using ESI
source. Mass spectra were acquired in centroid mode ranging from 200 to 2000 m/z. In the retention time range concerned (4.0 – 6.5 min) MS2 fragmentation on m/z related to flunarizine (405m/z) or respectively pimozide (462m/z) was executed. A 4 point calibration curve (0.001µg/mL – 1 µg/mL) was generated using blood serum of untreated mice as matrix for analytical standards. Concentration determination was performed based on peak area of appropriate fragment peaks. All experiments involving mice were reviewed and approved by Princeton University (protocol number 1930) and IACUCs. Human liver tissue for cell isolation was obtained from patients undergoing partial hepatectomy and after written informed consent was obtained.

**Statistical Analyses:** Data were analyzed in the statistical environment R (18). IC$_{50}$ values were computed using linear and logistic regression models implemented in the R packages drc (19) and lm. Pairwise comparisons of average treatment effects were assessed using Welch’s two-sample t-test, or differences to 100% control using the one-sample t-test. Due to small sample sizes (n<5), nonparametric tests could not be employed. Multiple-group comparisons and comparisons of dose-response-curves were performed using uni- and multivariate ANOVA. P-values are reported as (*) P ≤ 0.05; (**) P ≤ 0.01; (***) P ≤ 0.001. In figure 1, panel (A) shows means and standard deviations of 3 independent experiments. Panel (B) of 1-2 and Panel (C) of 2-4 independent experiments. Panel (D) shows means and standard deviations of a single experiment with 3 mice per group. Statistical significance between flunarizine and DMSO treatment was calculated by One Sample t-test in Panels (B) and (D) and Welch Two-sample t-test in Panel (C). In Figure 2, panels (A), (C) and (D) show means and standard deviation of 3 independent experiments. Panel (B) shows means and standard deviations from 3-7 independent experiments. Statistical significance between flunarizine and DMSO treatment was calculated by One Sample t-test. In Figure 3, Panel (B) shows means and standard deviation of 3 independent
experiments. Statistical significance between WT and each mutant response to flunarizine was calculated by ANOVA. Panel (D) shows mean values and standard deviations of 3 independent experiments. Statistical significance between WT and flunarizine-resistant mutant response against each antibody was calculated by ANOVA. In Figure 4, panel (B) shows means and standard deviation of at least 2 independent experiments. Panel (C) shows means and standard deviation of 3 independent experiments. Statistical significance between compound and DMSO treatment was calculated by Welch Two-sample t-test. In Figure 6, Panels (A) and (B) show means and standard deviation of 3 independent experiments. Panel (C) shows means and standard deviation of 5 independent experiments. Statistical significance between Jc1 WT and Jc1 flunarizine-resistant virus response to the indicated compounds in Panel (B) and between Jc1 WT and Jc1 flunarizine-resistant virus or genotype 5a in Panel (C) was calculated by ANOVA.

**HCV database search:** Globally sampled HCV E1 nucleotide sequences were downloaded and aligned according to overlying amino acid, as previously described (20). Frequencies of encoded amino acids at E1 positions 76 and 98 were calculated and plotted.
### Supporting Tables and Figures

#### Supporting Table S1: Panel of compounds tested for anti-HCV activity

<table>
<thead>
<tr>
<th>Compound</th>
<th>Main target</th>
<th>Use in clinics</th>
<th>IC50 (µM)</th>
<th>HCV Replication cycle</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amantadine</td>
<td>M2 influenza A protein antiviral</td>
<td></td>
<td>94.534</td>
<td>Assembly/Release</td>
</tr>
<tr>
<td>Amiodarone</td>
<td>beta-1 adrenergic receptors, Ca2+, Na+, and K+ channels</td>
<td>antiarrythmic/antianginal</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>BayK8644</td>
<td>Ca2+ channels</td>
<td></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Bepridil</td>
<td>Ca2+ channels</td>
<td>antianginal</td>
<td>3.7391</td>
<td>Assembly/Release</td>
</tr>
<tr>
<td>Diltiazem</td>
<td>Ca2+ channels</td>
<td>hypertension</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Disopyramide</td>
<td>Na+ channels</td>
<td>antiarrythmic</td>
<td>225.702</td>
<td>-</td>
</tr>
<tr>
<td>Dofetilide</td>
<td>K+ channels</td>
<td>antiarrythmic</td>
<td>58.0064</td>
<td>Entry/Assembly</td>
</tr>
<tr>
<td>Dronedarone</td>
<td>unknown</td>
<td>antiarrythmic</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Flecaainide</td>
<td>Na+ channels</td>
<td>antiarrythmic</td>
<td>16.6558</td>
<td>Release</td>
</tr>
<tr>
<td>Flunarizine</td>
<td>Ca2+ channels</td>
<td>migraine, vertigo, epilepsy, vertigo</td>
<td>0.38882</td>
<td>Entry</td>
</tr>
<tr>
<td>Fluphenazine</td>
<td>dopaminergic receptors calmodulin antagonist</td>
<td>antipsychotic</td>
<td>0.88493</td>
<td>Entry</td>
</tr>
<tr>
<td>Drug</td>
<td>Target (Receptors)</td>
<td>Effect</td>
<td>Time</td>
<td>Event</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------------------------------</td>
<td>-------------------------</td>
<td>------</td>
<td>----------------------</td>
</tr>
<tr>
<td>Methoxyverapamil</td>
<td>Ca&lt;sup&gt;2+&lt;/sup&gt; channels</td>
<td>antiarrhythmic</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>NB-DNJ</td>
<td>alpha-glucosidases</td>
<td>-</td>
<td>211.280</td>
<td>Assembly</td>
</tr>
<tr>
<td>Nifedipine</td>
<td>Ca&lt;sup&gt;2+&lt;/sup&gt; channels</td>
<td>hypertension</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>NN-DGJ</td>
<td>p&lt;sub&gt;7&lt;/sub&gt; protein</td>
<td>-</td>
<td>6.35686</td>
<td>Assembly</td>
</tr>
<tr>
<td>Pimozide</td>
<td>dopaminergic receptors</td>
<td>antipsychotic</td>
<td>0.551399</td>
<td>Entry/Assembly</td>
</tr>
<tr>
<td>Propafenone</td>
<td>Na&lt;sup&gt;+&lt;/sup&gt; channels, beta-1 adrenergic receptors</td>
<td>antiarrhythmic</td>
<td>1.7902</td>
<td>Assembly/Release</td>
</tr>
<tr>
<td>Quinidine</td>
<td>Na&lt;sup&gt;+&lt;/sup&gt; and K&lt;sup&gt;+&lt;/sup&gt; channels</td>
<td>antiarrhythmic</td>
<td>19.4551</td>
<td>Assembly/Release</td>
</tr>
<tr>
<td>R+IAA94</td>
<td>Cl&lt;sup&gt;-&lt;/sup&gt; channels</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Rimantadine</td>
<td>M2 influenza A protein</td>
<td>antiviral</td>
<td>55.2965</td>
<td>Assembly/Release</td>
</tr>
<tr>
<td>Sotalol</td>
<td>beta-1 adrenergic receptors, K&lt;sup&gt;+&lt;/sup&gt; channels</td>
<td>antiarrhythmic</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Trifluoperazine</td>
<td>dopaminergic receptors</td>
<td>antipsychotic, antiemetics</td>
<td>1.03444</td>
<td>Entry</td>
</tr>
<tr>
<td>Verapamil</td>
<td>Ca&lt;sup&gt;2+&lt;/sup&gt; channels</td>
<td>antiarrhythmic</td>
<td>7.36317</td>
<td>Assembly, Release</td>
</tr>
</tbody>
</table>
Supporting Table S2: Flunarizine IC$_{50}$ as calculated in different experimental setups

<table>
<thead>
<tr>
<th>Figures</th>
<th>Experiment</th>
<th>Calculated IC$_{50}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1A</td>
<td>Whole life cycle screen</td>
<td>0.38882</td>
</tr>
<tr>
<td>Figure 2A</td>
<td>4 hours infection with compound and medium change (12-well)</td>
<td>0.21941</td>
</tr>
<tr>
<td>Figure 3B</td>
<td>4 hours infection with compound and medium change (12-well)</td>
<td>0.14173</td>
</tr>
<tr>
<td>Sup. Figure 7A</td>
<td>4 hours infection with compound and medium change (96-well)</td>
<td>0.47752</td>
</tr>
</tbody>
</table>

Supporting Table S3: Prevalence of flunarizine resistance mutations among published HCV sequences

<table>
<thead>
<tr>
<th>Genotype 1 (n=6467)</th>
<th>E1 267</th>
<th>E1 289</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genotype 2 (n=340)</td>
<td>G=6457; E=2; R=8</td>
<td>Q=6459; R=4; K=2; T=1; L=1</td>
</tr>
<tr>
<td>Genotype 3 (n=564)</td>
<td>M=301; V=15; 10=A; 8=L; T=4; P=1; F=1</td>
<td>Q=339; H=1</td>
</tr>
<tr>
<td>Genotype 4 (n=83)</td>
<td>G=557; A=6; D=1</td>
<td>Q=563; L=1</td>
</tr>
<tr>
<td>Genotype 5 (n=238)</td>
<td>G=238</td>
<td>Q=238</td>
</tr>
<tr>
<td>Genotype 6 (n=176)</td>
<td>G=176</td>
<td>Q=176</td>
</tr>
</tbody>
</table>
Supporting Fig. S1: Selected compounds that inhibited HCV in the whole life cycle assay. Huh7-Lunet/hCD81/G-Luc cells constitutively expressing gaussia luciferase were transfected by electroporation with firefly luciferase reporter Jc1. After 4 h, medium containing 2-fold dilutions of the indicated compounds was added to the virus producing cells. Measurements of gaussia luciferase (cell viability) and firefly luciferase (replication) were taken after 48 h. The viruses produced were used to inoculate naïve Huh7-Lunet/hCD81/G-Luc cells and firefly luciferase was measured 48 h later (whole life cycle). Data shown are means and standard deviations of 3 independent experiments.

Supporting Fig. S2: Flunarizine does not inhibit assembly and release of infectious particles. (A) Extracellular and intracellular core levels and (B) extracellular and intracellular infectivity levels were measured in Jc1-transfected cells incubated for 48 h in presence of increasing concentrations of flunarizine (upper panels) or quinidine (lower panels) as an assembly control. Means and standard deviations of 2 independent experiments are given.

Supporting Fig. S3: Flunarizine does not inhibit infection by HCVpp carrying J6-derived E1-E2 proteins. Murine leukemia virus-based HCVpp bearing J6 E1E2 or VSV-G and transducing firefly reporter were used to inoculate cells in presence of 2-fold dilutions of flunarizine for 4 h or concanamycin A as a positive control given concentrations of flunarizine for 4 h. After washing and addition of medium, entry was measured by assessing firefly levels at 72 h post-infection. Means and standard deviations of 3 independent experiments are given.

Supporting Fig. S4: Flunarizine does not inhibit infection of Huh-7/hCD81/G-Luc cells by vesicular stomatitis virus (VSV) or by human Coronavirus 229E (HCoV-229E). Huh-7/hCD81/G-Luc cells were inoculated with firefly-luciferase expressing VSV or with renilla luciferase expressing HCoV-229 for 4 h in presence of 2-fold dilutions of flunarizine. Cells were washed and medium without compounds was added following inoculation. Virus replication was assessed.
by measuring firefly luciferase (VSV) or renilla luciferase levels (HCoV-229E) 24 h post-infection. Means and standard deviations of 3 independent experiments are given.

**Supporting Fig. S5:** GT2a (J6) derived E1-E2 genes are more susceptible to flunarizine than those of GT2b (J8) and E1 and E2 glycoproteins are important for flunarizine’s activity. (A) Given renilla luciferase reporter virus chimeras between J6 (GT2a) and J8 (GT2b) strains were created and their infectivity measured by infection of Huh7-Lunet/hCD81/G-Luc cells for 4 h (left side). These stocks were used to inoculate Huh7-Lunet/hCD81/G-Luc cells for 4 h with 2-fold dilutions of flunarizine (right side). (B) E1 and E2 renilla luciferase reporter chimera viruses between Jc1 (2a) and J8 (2b) were created and their infectivity measured by infection of Huh7-Lunet/hCD81/G-Luc cells for 4 h (left side). These stocks were used to inoculate Huh7-Lunet/hCD81/G-Luc cells for 4 h with 2-fold dilutions of flunarizine (right side). Renilla luciferase levels were assessed 48 h post-infection. Panel (A) shows mean values and standard deviations of 3 independent experiments. Panel (B) shows mean values and standard deviations of 3-4 independent experiments. Statistical significance between parental Jc1 and each chimeric virus in Panel (B) was calculated by ANOVA. (*) P ≤ 0.05; (**) P ≤ 0.01; (***) P ≤ 0.001.

**Supporting Fig. S6:** Flunarizine inhibits a post-attachment, late entry step and deletion of the HVR1 facilitates neutralization of HCV infection in suspension. (A) Huh7-Lunet/hCD81/G-Luc cells were exposed to given compounds prior to, during, or after virus inoculation. Note that heparin prevents virus attachment (5,21), anti-CD81 receptor antibodies (JS81) inhibit a post-binding step of HCV entry (5), BJ486K (BJ) acts on virus particles and also interferes with a post-attachment step of cell entry (8), whereas concanamycin A (ConA) and bafilomycin (Baf) both inhibit acidification of endosomes thus preventing low pH-triggered viral membrane fusion (22), a very late step of productive cell entry. Incubation with drugs was terminated by washing cells twice with PBS. Infection was quantified 48 h post inoculation by using luciferase assays.
(B) Parental Jc1 or Jc1 lacking the hypervariable region 1 (Jc1 DHVR1) was incubated with flunarizine, BJ486K or ConA at 37°C for 1h in suspension before 100-fold dilution and subsequent titration of virus infectivity by way of a limiting dilution assay. (C) Huh7-Lunet/hCD81/G-luc cells were incubated with F-Luc-Jc1 for 1h at 4°C to allow virus attachment but no downstream entry steps. Subsequently, unbound viruses were washed and inoculated cells were incubated at 37°C. Flunarizine, heparin, CD81-specific antibodies (JS81), or ConA were added either during inoculation (protocol 1) or every 20 min until 3 h after transfer to 37°C (protocol 2-7). Each compound was incubated with the cells for a total of 4 h before washing and medium exchange. Cells were lysed and firefly luciferase measured 48 h after inoculation. Values are normalized to DMSO control. Curves are plotted until maximum normalized infectivity of 100% was achieved. Panel (A) shows means and standard deviation of 2-4 independent experiments. Panel (B) shows means and standard deviation of 5 independent experiments. Panel (C) shows means and standard deviation of 3 independent experiments. Statistical significance between flunarizine and DMSO treatment was calculated by Welch Two-sample t-test in Panel (A) and One-sample t-test in Panel (B).

**Supporting Figure S7:** Flunarizine and related channel blockers have no effects on membrane fluidity. (A) Curcumin decreases membrane fluidity (23). DPH polarization increases in the presence of increasing concentrations of curcumin, both in 100% DOPC liposomes (open circles) or 37% cholesterol 63% DOPC liposomes (closed circles). (B) Flunarizine dihydrochloride (green), pimozide (orange), trifluoperazine dihydrochloride (dark blue) and fluphenazine hydrochloride (red) do not perturb membrane fluidity of 100% DOPC (open circles) or 37% cholesterol - 63% DOPC liposomes (closed circles). Note that the y-axis scales differ in (A) and (B). (C) and (D) The effect of curcumin varies with cholesterol content. The ratio of DPH depolarization in 37% cholesterol - 63% DOPC liposomes over that in 100% DOPC liposomes...
decreases at higher curcumin concentrations. The lack of effect of the channel blockers is not affected by cholesterol content. Averages ± ranges (too small to be seen at this scale for several data points) from two independent experiments. DPH emits fluorescent light of the same polarization as the excitation light in a rigid environment, whereas the emitted fluorescence becomes less polarized if DPH is in a fluid environment, in which it moves from the time of excitation to the time of emission. The lipophilic DPH inserts into lipid membranes. The degree of DPH fluorescence polarization in this environment is inversely related to membrane fluidity. As expected, DPH polarization increased with increasing concentration of curcumin (Supporting Figure S7 A) (23). In contrast, none of the channel blockers had any major effects on DPH fluorescence polarization (Supporting Figure S7B). Moreover, the effects of curcumin on membranes varied with the different levels of cholesterol in the liposomes. Curcumin induced a larger rigidification of cholesterol-free than of liposomes containing 37% cholesterol (Supporting Figure S7C). None of the channel blockers was affected by the presence of 37% or no cholesterol in the liposomes (Supporting Figure S7D).

Supporting Figure S8: Flunarizine and related channel blockers do not decrease cellular cholesterol. Huh7.5 cells were treated with test compounds for 24 h, or with β-cyclodextrin (BCD) for 3 h, before cellular lipids (and proteins) were extracted. Total cellular cholesterol levels are presented relative to protein. Trifluoperazine may increase cholesterol levels after 34 h, by less than 30%.

Supporting Figure S7S9: E1 and E2 proteins resistance mutations to flunarizine confer cross-resistance to pimozide. Huh7-Lunet/hCD81/G-Luc cells were inoculated with parental Jc1 renilla
reporter viruses (WT), or with Jc1-derivatives carrying the E2 resistance mutation alone (E2 M22TM405T), the two E1 resistance mutations alone (E1 M76V-M267V + E1 Q98H-Q289H) or a combination of these including the mutation in p7 (E1 M76V-M267V + E1 Q98H-Q289H + E2 M22T-M405T + p7 I7PI757T) for 4 h at 37°C in presence of 2-fold dilutions of either flunarizine or pimozide. Medium was changed and 48 h later cells were lysed and measured for renilla reporter activity. Means and standard deviations of 2 independent experiments are given.

References


Supplementary Figure 1

183x276mm (300 x 300 DPI)
Supplementary Figure 2

(A) Intracellular vs Extracellular Core fmoi/L (% control) with Flunarizine (µM)

(B) Intracellular vs Extracellular TCID₅₀ (% control) with Flunarizine (µM)

(C) Intracellular vs Extracellular Core fmoi/L (% control) with Quinidine (µM)

(D) Intracellular vs Extracellular TCID₅₀ (% control) with Quinidine (µM)
Supplementary Figure 5

(A) JFH-1

(B) JFH-1

Supplementary Figure 5
189x234mm (300 x 300 DPI)
Supplementary Figure 6

(A) % infection

(B) % infection

(C) 4 hours with compound

Supplementary Figure 6
188x257mm (300 x 300 DPI)
Supplementary Figure 7

(A) DPH polarization vs. [Cpd], μM
(B) DPH polarization ratio vs. [Cpd], μM

Supplementary Figure 7
169x191mm (300 x 300 DPI)

- Curcumin
- Flunarizine
- Pimozide
- Trifluoperazine
- Fluphenazine
Supplementary Figure 9

% infection

Supplementary Figure 9
189x108mm (300 x 300 DPI)